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## Multianalyte Microspot Immunoassay—Microanalytical "Compact Disk" of the Future

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Throughout the 1970s, controversy centered both on immunoassay "sensitivity" per se and on the relative sensitivities of labeled antibody (Ab) and labeled analyte methods. Our theoretical studies revealed that RIA sensitivities could be surpassed only by the use of very high-specific-activity nonisotopic labels in "noncompetitive" designs, preferably with monoclonal antibodies. The time-resolved fluorescence methodology known as DELFIA—developed in collaboration with LKB/Wallac—represented the first commercial "ultrasensitive" nonisotopic technique based on these theoretical insights, the same concepts being subsequently adopted in comparable methodologies relying on the use of chemiluminescent and enzyme labels. However, high-specific-activity labels also permit the development of "multianalyte" immunoassay systems combining ultrasensitivity with the simultaneous measurement of tens, hundreds, or thousands of analytes in a small biological sample. This possibility relies on simple, albeit hitherto-unexploited, physicochemical concepts. The first is that all immunoassays rely on the measurement of Ab occupancy by analyte. The second is that, provided the Ab concentration used is "vanishingly small," fractional Ab occupancy is independent of both Ab concentration and sample volume. This leads to the notion of "ratiometric" immunoassay, involving measurement of the ratio of signals (e.g., fluorescent signals) emitted by two labeled Abs, the first (a "sensor" Ab) deposited as a microspot on a solid support, the second (a "developing" Ab) directed against either occupied or unoccupied binding sites of the sensor Ab. Our preliminary studies of this approach have relied on a dual-channel scanning-laser confocal microscope, permitting microspots of area  $100\ \mu\text{m}^2$  or less to be analyzed, and implying that an array of  $10^6$  Ab-containing microspots, each directed against a different analyte, could, in principle, be accommodated on an area of  $1\ \text{cm}^2$ . Although measurement of such analyte numbers is unlikely ever to be required, the ability to analyze biological fluids for a wide spectrum of analytes is likely to transform immunodiagnostics in the next decade.

**Additional Keyphrases:** *ratiometric immunoassays · scanning-laser confocal microscope · fluoroimmunoassay*

Immunoassay and other protein-binding assay methods based on the use of radioisotopic labels have played a major role in medicine during the past three decades.

Their utility and importance have derived primarily from the structural specificity of many reactions between binding proteins and analytes and the detectability of isotopically labeled reagents, the latter endowing such techniques with "exquisite sensitivity." Recently, however, interest has increasingly focused on nonisotopic techniques based on identical analytical principles, differing only in the nature of the marker used to label the reactant (e.g., antibody or antigen), whose distribution between reacted ("bound") and unreacted ("free") fractions constitutes the assay "response."

The basic aims underlying this interest can be broadly classed under four main headings:

- avoidance of the environmental, legal, economic, and practical disadvantages of isotopic techniques (e.g., limited shelf life of isotopically labeled reagents, problems of radioactive waste disposal, cost and complexity of radioisotope counting equipment), particularly those impeding the development of, for example, simple diagnostic kits for home or doctor's office use;
- achievement of greater assay sensitivity;
- "direct" measurement of analyte concentrations by use of transducer-based "immunosensors";
- simultaneous measurement of multiple analytes ("multianalyte assay").

In this presentation I will focus primarily on the last of these objectives, using this to set out the principles underlying our present attempts to develop a new "miniaturized" technology that will permit the simultaneous measurement of an unlimited number of analytes in a small biological sample such as a single drop of blood. However, retention (and, if possible, improvement) of the high sensitivities of conventional isotopic techniques is a basic aim not only of our own studies in this area but also of most other endeavors falling under the above headings. It is therefore appropriate to preface this paper with a discussion of the general principles underlying the attainment of high binding-assay sensitivity.

### Immunoassay Sensitivity: Some Basic Concepts

#### Definition of Assay Sensitivity

The need to establish assay conditions yielding maximal sensitivity underlay the independent construction of mathematical theories of immunoassay design by both Yalow and Berson (1) and Ekins et al. (2) in the course of the original development of these methods in the early 1960s. Regrettably, these theoretical studies led to a prolonged controversy, arising largely from the conflicting concepts of "sensitivity" adopted by the two groups (see Figure 1). Briefly, Berson and Yalow, in their many publications relating to immunoassay design (e.g., 1, 3), defined sensitivity as the slope of the

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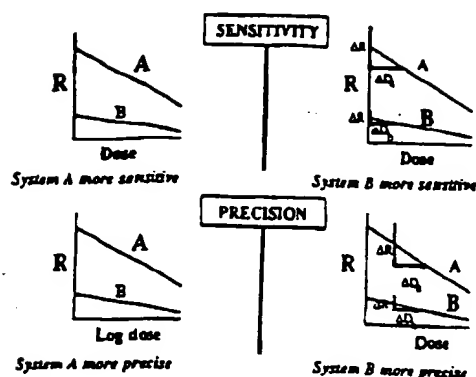


Fig. 1. The differing concepts of sensitivity and precision underlying radioimmunoassay design theories developed by (left) Yalow and Berson (e.g., 1, 3) and (right) Ekins et al. (2, 4)

Yalow and Berson define assay A as more sensitive because it yields a response curve of greater slope. Ekins et al. define assay B as more sensitive because the imprecision of measurement of zero dose ( $\sigma_0$ ) is less. Yalow and Berson likewise define an assay system as more precise if it yields a steeper response curve when data are plotted on a log dose scale

response curve relating the fraction or percentage of labeled antigen bound ( $b$ ) to analyte concentration ( $[H]$ ). In contrast, Ekins et al. (e.g., 2, 4) defined sensitivity as the (im)precision of measurement of zero dose, this quantity being indicative of, and essentially equivalent to, the lower limit of detection.

The key difference between these two definitions clearly lies in the dependence of the assay detection limit on the error (imprecision) in the measurement of the response variable. By neglecting this crucial factor, the "response curve slope" definition leads to many obvious absurdities. For example, plotting conventional RIA data in terms of the response metameter  $B/F$  (i.e., the bound to free ratio) suggests that assay "sensitivity" is increased by increasing the antibody concentration in the system; however, the converse conclusion is reached if identical data are plotted in terms of  $F/B$  (see Figure 2). Observation of the shape and slopes of response curves without detailed error analysis thus constitutes a totally misleading guide to optimal immunoassay design. This approach has, however, characterized many of the studies conducted in the immunoassay field during the past 30 years, and has been the source of much

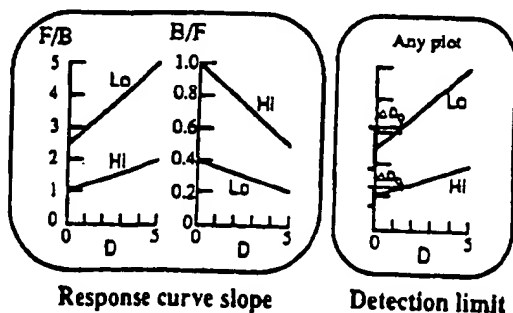


Fig. 2. Schematic representation of RIA dose-response curves observed for high and low antibody concentrations plotted in terms of (left) the free/bound fraction ( $F/B$ ); (center) the bound/free fraction ( $B/F$ )

Note that the low antibody concentration yields a response curve of greater slope when the assay response is plotted in terms of  $F/B$ , but of lower slope when plotted in terms of  $B/F$ . The precision of measurement of zero dose ( $\Delta D_0$ ) is independent of the coordinate frame used to plot assay data (see right)

mythology. For example, consideration of the Law of Mass Action reveals that, when response curves corresponding to different antibody concentrations are plotted in terms of  $b$  vs  $[H]$ , the maximal slope at zero dose is obtained for a concentration of  $0.5/K$  (where  $K$  is the affinity constant), in which circumstance the zero dose response ( $b_0$ ) is 33%. This conclusion led to Berson and Yalow's enunciation of the well-known dictum (which, albeit erroneous, is broadly adhered to by many immunoassay practitioners and kit manufacturers) that, to maximize RIA sensitivity, the amount of antibody to use in the system is that which binds 33% of labeled antigen in the absence of unlabeled antigen (1, 3).

Disagreement regarding the concept of sensitivity inevitably led to prolonged dispute regarding immunoassay design (5). However, although it is still common to encounter publications in the field that rely solely on the response curve slope as a measure of sensitivity, the assay detection limit is now widely accepted as the only valid indicator of this parameter, and we do not therefore intend to dwell further on this issue here. It is nevertheless relevant to an understanding of the "miniaturized" assay methodology described below to emphasize that untenable concepts of both sensitivity and precision underlie many of the commonly accepted rules governing current immunoassay-design practice, some of which are contravened in our own approach.

#### Basic Immunoassay Designs

It is likewise important in the present context to comprehend the basis of the various types of immunoassays currently in use, and the constraints on the sensitivities of which they are potentially capable. The radioimmunoassay and analogous protein-binding assay techniques originally developed for the measurement of insulin by Yalow and Berson (6), and of thyroxine and vitamin  $B_{12}$  by Ekins and Barakat (7, 8), relied on the use of a labeled analyte marker to reveal the products of the binding reactions between analyte and binder (Figure 3, left). This approach has subsequently often been portrayed as relying on "competition" between labeled and unlabeled analyte molecules for a limited number of protein-binding sites, such assays being frequently referred to as "competitive."

Subsequently, Wide et al. in Sweden (9), followed shortly by Miles and Hales in the U.K. (10), developed labeled antibody methods (Figure 3, right). These methods represented an extension of the "labeled reagent" methods (utilizing radiolabeled organic compounds such as  $^{125}\text{I}$ -labeled  $p$ -iodosulfonyl chloride,  $^3\text{H}$ acetic anhydride, and other similar reagents) devised, during the early 1950s, by Keston et al. (11), Avivi et al. (12), and others for quantifying amino acids, steroid and thyroid hormones, etc. Although radiolabeled antibody methods (immunoradiometric assays; IRMAs) were originally claimed (13) to be more sensitive than methods based on the use of radiolabeled analyte, these claims were supported by neither rigorous theoretical analysis nor persuasive experimental evidence, and for some time remained controversial. Further doubt on their validity

analyte + antibody\*  $\rightleftharpoons$  analyte : antibody\* (B)  
 + residual analyte  
 + residual antibody\* (F)

Measure "fraction bound" (B) :  $[Ab]_{\text{bound}} \rightarrow \infty$   
 Measure "fraction free" (F) :  $[Ab]_{\text{free}} \rightarrow 0$

Fig. 3. Labeled-analyte (left) and labeled-antibody (right) assay systems compared

Labeled-analyte assay systems essentially rely on observation of an analyte "marker" to reveal the products of the reaction between analyte and antibody (although the labeled analyte is not necessarily identical to the unlabeled analyte in its binding characteristics vis-à-vis antibody). Note that, irrespective of which fraction of the labeled analyte is measured after the binding reaction, the optimal antibody concentration required to maximize sensitivity in such a system tends toward zero (assuming a background signal of 0). Labeled-antibody systems rely on observation of an antibody "marker" to reveal the products of the binding reaction between analyte and antibody. In this case, the optimal antibody concentration required to maximize sensitivity tends toward zero when the "free" antibody fraction is measured, but tends toward infinity when the bound fraction is determined (likewise assuming zero background).

was cast by the publication by Rodbard and Weiss in 1973 (14) of detailed theoretical studies demonstrating that both labeled analyte and labeled antibody methods possessed essentially equal sensitivities. (Note: These authors suggested that IRMAS might be more sensitive in the assay of small polypeptides, in which radioiodine incorporation into the antigen molecule was restricted; conversely, these assays would be less sensitive for the measurement of antigens of high molecular mass.) Nevertheless, despite the appearance of this publication, the belief that labeled antibody methods per se are intrinsically more sensitive than the corresponding labeled analyte methods gained wide acceptance among clinical chemists.

The reason for confusion on this issue is that the greater potential sensitivity of certain assay formats is not really a consequence of the labeling of antibody as opposed to analyte; indeed, the apparent antithesis between labeled-analyte and labeled-antibody methods diverts attention from the true reasons underlying the superior sensitivity of certain assay designs. Theoretical analysis (see, e.g., 4, 15) reveals that, assuming "perfect" separation of the products of the binding reaction (i.e., no misclassification of bound and free moieties), the optimal antibody concentration (for maximal sensitivity) in a labeled analyte immunoassay invariably tends to zero, irrespective of whether the free or bound labeled analyte fraction is measured, whereas in labeled-antibody methods the optimal antibody concentration depends on which labeled-antibody fraction is measured (see Figure 3). If the free (unreacted) antibody fraction is measured, the optimal concentration also tends to zero; conversely, if the analyte-bound fraction is measured, the concentration tends to infinity. In short, of the four basic measurement strategies available—labeled analyte, with measurement of free or bound reaction product, and labeled antibody, also with measurement of free or bound product—only one permits, in practice, the use of antibody concentrations approaching infinity.

This particular approach may, for want of a better term, be described as "noncompetitive," although it must be emphasized that such terminology involves a departure from the original meanings attached to "competitive" and "noncompetitive" when these descriptions were first used in the present context. Indeed, as discussed below, assays may be subclassified in this manner when no labeled reagent of any kind is involved.

However, the categorization of immunoassays and other binding assays as competitive or noncompetitive, depending on the binding agent concentration yielding maximal assay sensitivity, itself obscures the underlying reasons for the existence of this divergence in assay designs, and may thus be misleading. These reasons may be more readily understood if the basic principles of such assays are portrayed differently from their customary presentation.

#### The "Antibody Occupancy Principle" of Immunoassay

When a "sensor" antibody is introduced into an analyte-containing medium, binding sites on the antibody are occupied by analyte molecules to a fractional extent that reflects both the equilibrium constant governing the binding reaction, and the final concentration of free analyte present in the mixture. This proposition stems immediately from the Law of Mass Action, which can be written as

$$[AbAg]/[fAb] = K[fAg] \quad (1)$$

or as fractional occupancy of antibody binding sites, given by

$$[AbAg]/[Ab] = K[fAg]/(1 + K[fAg]) \quad (2)$$

where  $[AbAg]$ ,  $[Ab]$ ,  $[fAb]$ , and  $[fAg]$  represent the concentrations (at equilibrium) of bound and total antibody, and free antibody and antigen (analyte), respectively, and  $K$  = equilibrium constant. The final concentration of free analyte generally depends on the concentrations of both total analyte and antibody; however, when total antibody approximates  $0.05/K$  or less, free and total antigen ( $[Ag]$ ) concentrations do not differ significantly, and fractional occupancy of antibody is given by

$$[AbAg]/[Ab] = K[Ag]/(1 + K[Ag]) \quad (3)$$

Assays utilizing this concept have been termed "ambient analyte immunoassays" (16), fractional occupancy being independent of both sample volume and antibody concentration (see below).

All immunoassays essentially depend on measurement of the "fractional occupancy" of the sensor antibody after its reaction with analyte (see Figure 4). Techniques relying on the measurement of unoccupied antibody binding sites (from which antibody occupancy is implicitly deduced by subtraction) necessitate—for attainment of maximal sensitivity—the use of sensor antibody concentrations tending to zero; these assays

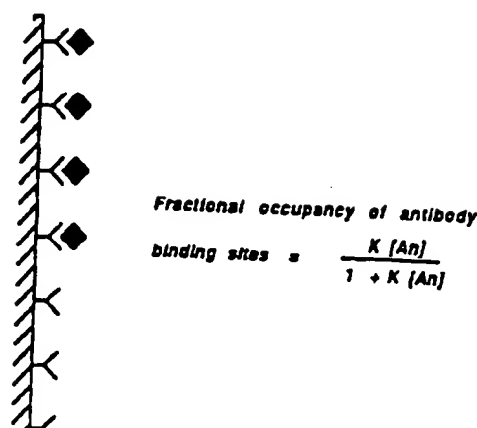


Fig. 4. The antibody binding-site occupancy principle of immunoassay  
All immunoassays implicitly rely on the measurement of (fractional) binding-site occupancy by analyte

may therefore be categorized as "competitive." Conversely, techniques in which occupied sites are *directly* measured permit (in principle) the use of relatively high concentrations of sensor antibody and may be described as "noncompetitive." This difference in assay design simply reflects the proposition that, to minimize error in the measurement, it is generally undesirable to measure a small quantity by estimating the difference between two large quantities.

These concepts are illustrated in Figure 5, which portrays basic immunoassay formats currently in common use. Conventional RIA and other similar "labeled-analyte" techniques rely on measurement of *unoccupied* sites, generally by back-titration (either simultaneous or sequential) with labeled analyte, but anti-idiotypic antibody (reactive only with unoccupied sites on the sensor antibody) may be used for the same purpose. In the case of single-site labeled-antibody assays, the labeled antibody itself constitutes the sensor antibody; after reaction with analyte, this sensor antibody may be separated into occupied and unoccupied fractions through use of (e.g.) an immunosorbant (comprising antigen, antigen analog, or anti-idiotypic antibody linked to a solid support). If, after separation, the "signal" emitted by labeled antibody *bound* to analyte (i.e., the "occupied" fraction) is measured directly, the assay can be classed as "noncompetitive." Conversely, if one measures the labeled antibody *not bound* to analyte (i.e., that attached to the immunosorbant), then the assay is "competitive."

Two-site "sandwich" assays are clearly more complex because they rely on two antibodies and can be considered from two points of view. For our present purposes, the solid-phase antibody can be regarded as the "sensor" antibody, with the labeled antibody enabling the occupied sensor-antibody binding sites to be distinguished. Seen from this viewpoint, two-site assays may be classed as "noncompetitive."

These considerations emphasize that the differences in design distinguishing so-called competitive and non-competitive methods are essentially unrelated to which

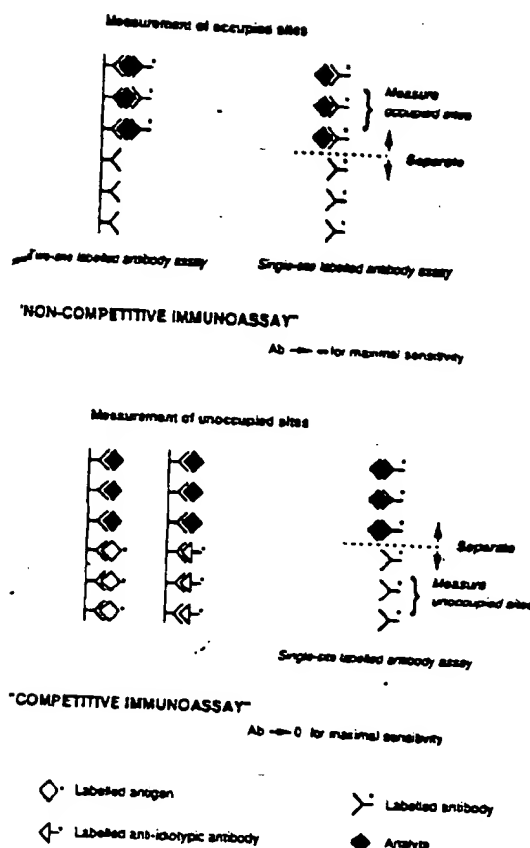


Fig. 5. Basic competitive and noncompetitive immunoassay designs  
The distinction between noncompetitive and competitive immunoassays reflects the way in which antibody binding-site occupancy is observed. Labeled-antibody methods are "noncompetitive" if occupied sites of the (labeled) antibody are directly measured, but are "competitive" (lower right) when unoccupied sites are measured. Labeled-antigen (lower left) or labeled-anti-idiotypic-antibody methods (lower center) rely on measurement of sites unoccupied by analyte, and are therefore of "competitive" design

component (if any) of the reaction system is labeled. Indeed, in the case of transducer-based "immunosensors," no component is labeled; nevertheless, the design of the immunosensor will differ significantly, depending on whether a measurable signal is yielded by occupied or unoccupied antibody binding sites situated on its surface. In short, the terms "competitive" and "noncompetitive" merely reflect alternative approaches to the determination of the occupancy of antibody binding sites and lead to differences in the optimal antibody concentration required to minimize the effects of random errors arising in the determination.

Competitive and noncompetitive immunoassays can be shown to differ significantly in many of their performance characteristics, including their sensitivities. In both types of assays, both the affinity constant ( $K$ ) of the antibody and the specific activity of the label are important in determining sensitivity; however, in practice, the sensitivity of competitive assays is primarily limited by the affinity constant of the antibody, whereas the specific activity of the label is more important in non-competitive systems. In both cases, the "experimental" or "manipulation" error in the measurement of the zero-dose response ( $R_0$ ) [i.e., the relative error ( $\sigma_{R_0}/R_0$ ) arising from pipetting and other operations, but not including the statistical signal measurement error per

se] is of key importance in determining "potential" assay sensitivity (i.e., the sensitivity obtained by assuming the specific activity of the label to be infinite, implying zero error in signal measurement). Thus the potential sensitivity of a competitive assay can be shown to be  $\sigma_R/KR_0$ , whereas that of a noncompetitive assay is given by  $R_0\sigma_R/[Ab]KR_0$ , where, in the latter case,  $R_0$  is assumed to represent the labeled antibody misclassified as bound ( $[bAb]_0$ ), commonly referred to as "nonspecifically bound" antibody. Thus  $R_0/[Ab] = f$ , the fraction of labeled antibody that is nonspecifically bound, and  $R_0\sigma_R/[Ab]KR_0 = f\sigma_R/KR_0$ . Assuming that the relative error ( $\sigma_R/R_0$ ) in the measurement of the zero-dose response is approximately identical for both competitive and noncompetitive assays, it is evident from this simple analysis that the potential sensitivity of noncompetitive methods is greater than that of competitive methods by the factor  $f$ , i.e., by the fraction of labeled antibody that is "nonspecifically bound." For example, if the nonspecifically bound fraction is 0.01%, a noncompetitive strategy is potentially capable of a sensitivity 10 000-fold greater than that of a competitive approach, other factors being equal.

These findings are summarized in Figure 6 (left), which shows the relationships between sensitivity (expressed in terms of molecules per milliliter) and anti-

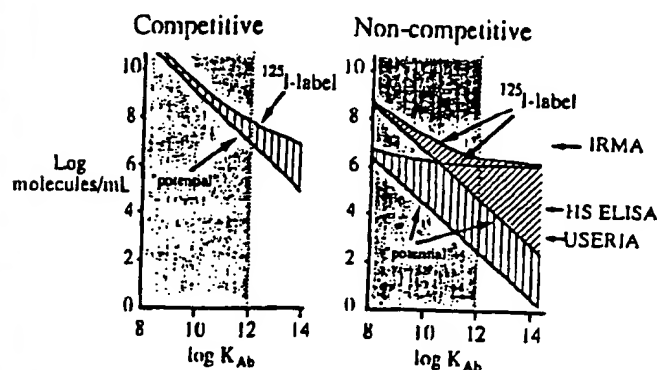


Fig. 6. Theoretically predicted sensitivities of competitive and non-competitive immunoassay methods (represented by the SD of zero analyte measurements, expressed as molecules/mL) plotted as a function of antibody affinity ( $K$ )

Note: in noncompetitive sandwich assays, the antibody affinity referred to is that of the labeled antibody. In the competitive assays, calculations are based on the assumption that the experimental error (CV) incurred in the measurement of the assay response (e.g., fraction of labeled antigen bound) is 1%. The "potential sensitivity" curve assumes the use of a label of infinite specific activity, implying that the error in the measurement of the label per se is zero. The  $^{125}\text{I}$ -label curve indicates the loss in sensitivity arising from the statistical error incurred in counting  $^{125}\text{I}$  disintegrations for a finite counting time. Note that, if using antibodies with an affinity  $<10^{12}$  L/mol (the maximum achieved in practice), little increase in sensitivity can be achieved by using labels of higher specific activity than  $^{125}\text{I}$ . For noncompetitive assays, the potential sensitivity curves shown relate to values of nonspecific binding of labeled antibody of 1% (upper curves) and 0.01% (lower curves), and emphasize the improvement in sensitivity potentially attainable by minimizing nonspecific binding. The corresponding  $^{125}\text{I}$ -label curves demonstrate the much greater loss in sensitivity (compared with that potentially attainable) when a radiolabel is used, and the special advantages of nonisotopic labels of higher specific activity in noncompetitive assay designs (particularly if nonspecific binding is reduced to 0.1% or less). Arrows indicate assay sensitivities reported for noncompetitive immunoassays based on  $^{125}\text{I}$  (IRMA), and enzymes relying on fluorogenic (HS-ELISA) (28) and radioactive (USERIA) (29) substrates. These conclusions underlay the original development (19, 20) of time-resolved fluorimmunoassay (DELFA), the first nonisotopic "ultra-sensitive" immunoas-

body affinity in an optimized competitive (labeled analyte) assay. For this analysis, we assume (a) the use of a label of infinite specific activity, and (b) the use of  $^{125}\text{I}$  as a label, the radioactivity of the samples being counted for 1 min. Computations of the theoretically optimal reagent concentrations (on which calculations represented in Figure 6 rely) were based on the further assumptions that (c) the radioactivity of the antibody-bound labeled-analyte fraction was counted and (d) the (relative) "experimental error" component in the measurement of the bound fraction ( $\sigma_b/b$ ) was 1%. Given these assumptions, the "potential" sensitivity attainable in such an assay is  $\sigma_b/Kb$ , where  $K$  is the affinity constant of the antibody. [For example, if the affinity constant is  $10^{12}$  L/mol, and  $\sigma_b/b$  is 0.01 (1%), maximal assay sensitivity is  $10^{-14}$  mol/L, or  $\sim 6 \times 10^6$  molecules/mL.] The additional "signal measurement error" arising in consequence of counting radioactive samples for a finite time implies a loss of assay sensitivity, as shown by the upper curve in Figure 6 (left). However, the resulting loss in sensitivity is relatively small for antibodies of affinities  $<10^{12}$  L/mol, and is negligible for antibodies with affinities  $<10^{11}$  L/mol. In other words, if the assayist can accept individual sample counting times of 1–5 min, little improvement in sensitivity is gained by using alternative labels of higher specific activities than  $^{125}\text{I}$ . However, similar considerations suggest that radioisotopic labels of much lower specific activity than  $^{125}\text{I}$  (e.g.,  $^3\text{H}$ ) may limit the sensitivities of the assays (such as steroid assays) in which they are used, notwithstanding the use of relatively long sample counting times.

The other main conclusions stemming from such analysis are the importance of both minimizing "manipulation" errors and using antibodies of high binding affinity. For example, an increase in  $\sigma_b/b$  to 3% implies an approximate threefold loss in sensitivity, notwithstanding the fact that an assay reoptimized in response to the deterioration in operator skill that these numbers imply would utilize less antibody and labeled analyte, thereby partially offsetting the consequences of poor pipetting. But the most important conclusion emerging from the analysis is the near impossibility, in practice, of achieving immunoassay sensitivities better than about  $10^7$  molecules/mL by using a competitive approach, irrespective of the nature of the label used, if one assumes an upper limit to antibody binding affinities on the order of  $10^{12}$  L/mol.

The results of a similar analysis of the sensitivity limitations applying to noncompetitive (two-site) assays (15) are illustrated in Figure 6 (right). Two sets of curves are portrayed here, corresponding to the assumptions of 1% and 0.01% nonspecific binding of labeled antibody to the capture-antibody substrate. Such analysis likewise yields important conclusions relevant to assay design, e.g., the crucial importance of reducing nonspecific binding of labeled antibody to an absolute minimum. Furthermore, if nonspecific binding is reduced to  $\sim 0.01\%$ , just as high sensitivity is achievable



by using an antibody of  $K = 10^8$  L/mol in an optimized noncompetitive assay design as by using an antibody of  $K = 10^{12}$  L/mol in a competitive method. One of the most important conclusions is that the sensitivities potentially attainable with high-affinity antibodies ( $K > 10^{10}$  L/mol) are beyond the reach of radioisotopically based methods, which (because of the relatively low specific activities of isotopes such as  $^{125}\text{I}$ ) are limited in practice to sensitivities of the order of  $10^6$ – $10^7$  molecules/mL or more. In short, although, under certain circumstances, noncompetitive IRMAs may be somewhat more sensitive than corresponding RIA techniques (assuming the use of the same antibody in each methodology), the potential advantages (*vis-à-vis* sensitivity) of the noncompetitive approach can be realized only by using nonisotopic labels of much higher specific activity than  $^{125}\text{I}$ . The superiority of such labels is most apparent when they are combined with high-affinity antibodies; however, Figure 6 demonstrates that, even with use of antibodies with affinities of about  $10^8$ – $10^9$  L/mol, nonisotopic labels may yield a substantial improvement in sensitivity.

These theoretical conclusions, together with the publication by Köhler and Milstein (18) of methods of *in vitro* production of monoclonal antibodies (1), constituted the basis of my laboratory's collaborative development (initiated around 1976) with the instrument manufacturer LKB/Wallac of the time-resolved fluorometric immunoassay methodology now known as DELFIA (19, 20). This methodology was the first "ultra-sensitive" nonisotopic immunoassay methodology to be developed. The same basic approach has subsequently been adopted by many other manufacturers, using a variety of high-specific activity labels (Table 1).

Against this background, let us now turn to the development of highly sensitive, miniaturized "microspot" immunoassays and multianalyte assay systems.

### Antibody "Microspot" Immunoassay: Basic Concepts and Theory

#### Ambient Analyte Immunoassay

Particular attention has been drawn above to the specious notion that an antibody concentration approximating  $0.5/K$  is required to maximize the sensitivity of conventional labeled-antigen assays. This proposition is implicitly overturned by the development of "microspot" immunoassays, which we expect to provide the basis of a new generation of binding assay methods. But before

discussing this methodology in detail, another basic analytical concept must be examined.

The recognition that all immunoassays essentially rely on measurement of antibody occupancy leads to a potentially important type of assay, ambient analyte immunoassay (16). This name is intended to describe assay systems that, unlike conventional methods, measure the analyte concentration in the medium to which an antibody is exposed, being independent both of sample volume and of the amount of antibody present. The possibility of developing such assays follows from the Law of Mass Action, which leads to the following equation, representing the fractional occupancy ( $F$ ) by analyte of antibody binding sites (at equilibrium):

$$F^2 - F\{([A]/[Ab]) + ([An]/[Ab]) + 1\} + [An]/[Ab] = 0 \quad (4)$$

where  $[An]$  = analyte concentration,  $[Ab]$  = antibody concentration (both in units of  $1/K$ ).<sup>1</sup>

From this equation it may readily be shown that, for antibody concentrations approaching 0,  $F \approx [An]/(1 + [An])$ . This conclusion is illustrated in Figure 7, in which the fractional occupancy of ("monospecific" or "monoclonal") antibody binding sites in the presence of various analyte concentrations is plotted against antibody concentration. When an antibody concentration of less than (say)  $0.01/K$  (the antibody preferably, but not essentially, being coupled to a solid support) is exposed to an analyte-containing medium, the resulting (fractional) occupancy of antibody binding sites solely reflects the ambient concentration of analyte<sup>1</sup> and is independent of the total amount of antibody in the system. (If, for example,  $K = 10^{11}$  L/mol, an antibody binding-site concentration of  $0.01/K$  represents  $0.01 \times 10^{-11}$  mol/L, or  $6.02 \times 10^7$  binding sites/mL.) Analyte binding by antibody causes depletion of (unbound) analyte in the medium but, because the amount bound is small, the resulting reduction in the ambient concentration of analyte is insignificant. For example, if the concentration of binding sites of the sensor antibodies is  $< 0.01/K$ , analyte depletion in the medium is invariably  $< 1\%$ , and the system is therefore effectively indepen-

<sup>1</sup> Expression of reagent concentrations in terms of  $1/K$  units has the effect of generalizing the graphical representation of binding assay data. The terms  $[Ab]$  and  $[An]$  are underlined to indicate that this convention has been adhered to in deriving equation 4. They do not refer to molar concentrations and are not interchangeable with  $[Ab]$  and  $[An]$ . For example, if the antibody possesses an affinity (constant) for analyte of  $10^{11}$  L/mol, a concentration of  $10^{-11}$  mol/L (represented in units of  $1/K$ ) is 1 (dimensionless) unit. Thus, fractional occupancy curves based on equation 4 are identical for all antibodies if this way of expressing antibody concentration is adopted: i.e., curves relating  $F$  to analyte concentration will be identical for systems using  $10^{-11}$  mol/L concentrations of an antibody with an affinity of  $10^{11}$  L/mol,  $10^{-10}$  mol/L of an antibody with an affinity of  $10^{10}$  L/mol,  $10^{-9}$  mol/L of an antibody with an affinity of  $10^9$  L/mol, etc. (provided the analyte concentration is expressed in the same manner).

<sup>2</sup> The term "ambient" is used to indicate that antibody occupancy reflects the analyte concentration to which antibody binding sites are exposed, not the amount of analyte in the incubation tube; i.e., the system is independent of sample volume.

Table 1. Detection Limits According to Type of Label

Label	Specific activity
$^{125}\text{I}$	1 detectable event per second per $7.5 \times 10^6$ labeled molecules
Enzyme label	Determined by enzyme "amplification factor" and detectability of reaction product
Chemiluminescent label	1 detectable event per labeled molecule
Fluorescent label	Many detectable events per labeled molecule

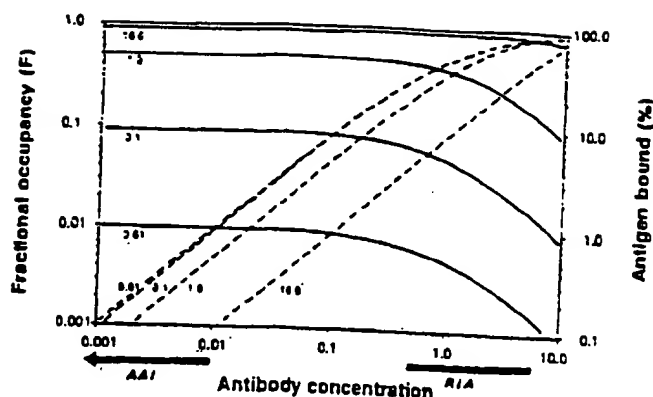


Fig. 7. Fractional antibody binding-site occupancy ( $F$ , see equation 4) plotted as a function of antibody binding-site concentration for different values of analyte (antigen) concentration (—), and the percentage binding ( $b$ ) of analyte to antibody (right-hand ordinate; - -).

All concentrations are expressed in units of  $1/K$ . Note that for antibody concentrations  $< 0.01/K$  (approximately), the percentage binding of analyte is  $< 1\%$  for all analyte concentrations, and fractional binding-site occupancy is essentially unaffected by variations in antibody concentration extending over several orders of magnitude, being governed solely by antigen concentration (ambient analyte immunoassay). Note that radioimmunoassays and other "competitive" immunoassays are conventionally designed to use antibody concentrations approximating  $0.5/K$ – $1/K$  or more (implying binding of analyte concentrations tending to zero ( $b_0$ )  $> 30\%$ ), in accordance with the precepts of Yalow and Berson (1, 3).

dent of sample volume.

These conclusions lead to two further concepts. First, the antibody may be confined to a "microspot" on a solid support, such that the total number of antibody binding sites within the microspot is  $< v/K \times 10^{-5} \times N$ , where  $v$  = the sample volume to which the microspot is exposed (in milliliters) and  $N$  = Avogadro's number ( $6 \times 10^{23}$ ). For example, if  $v = 1$  and  $K = 10^{12}$  L/mol, then the

maximum number of binding sites that will cause negligible disturbance ( $< 1\%$ ) to the ambient concentration of analyte is  $6 \times 10^6$ , this number being greater for lower-affinity antibodies. Furthermore, the perception that the ratio of occupied (or unoccupied) sites to total binding sites is solely dependent on the ambient concentration of analyte leads to the concept of a dual-label, "ratiometric," microspot immunoassay.

### Dual-Label Microspot Immunoassay

After exposure of a microspot of antibody (located on a suitable probe) to an analyte-containing fluid (see Figure 8, left), the probe may be removed and exposed to a solution containing a high concentration of a "developing" antibody directed against either a second epitope (i.e., the occupied site) on the analyte molecule if the molecule is large, or against unoccupied binding sites on the antibody in the case of small analyte molecules (Figure 8, right). The fractional occupancy of the sensor antibody may thus be estimated by measuring the ratio of sensor and developing antibodies that form the dual-antibody "couplets." This can be readily achieved by labeling the sensor and the developing antibodies with different labels, e.g., a pair of radioactive, enzyme, or chemiluminescent markers (or even labels of entirely different nature). Fluorescent labels are potentially particularly useful in this context because, by the use of optical scanning techniques (Figure 9), they permit the scanning of arrays of antibody "microspots" distributed over a surface (each microspot directed against a different analyte), so that multiple analyte assays may be performed simultaneously on the same sample. Several

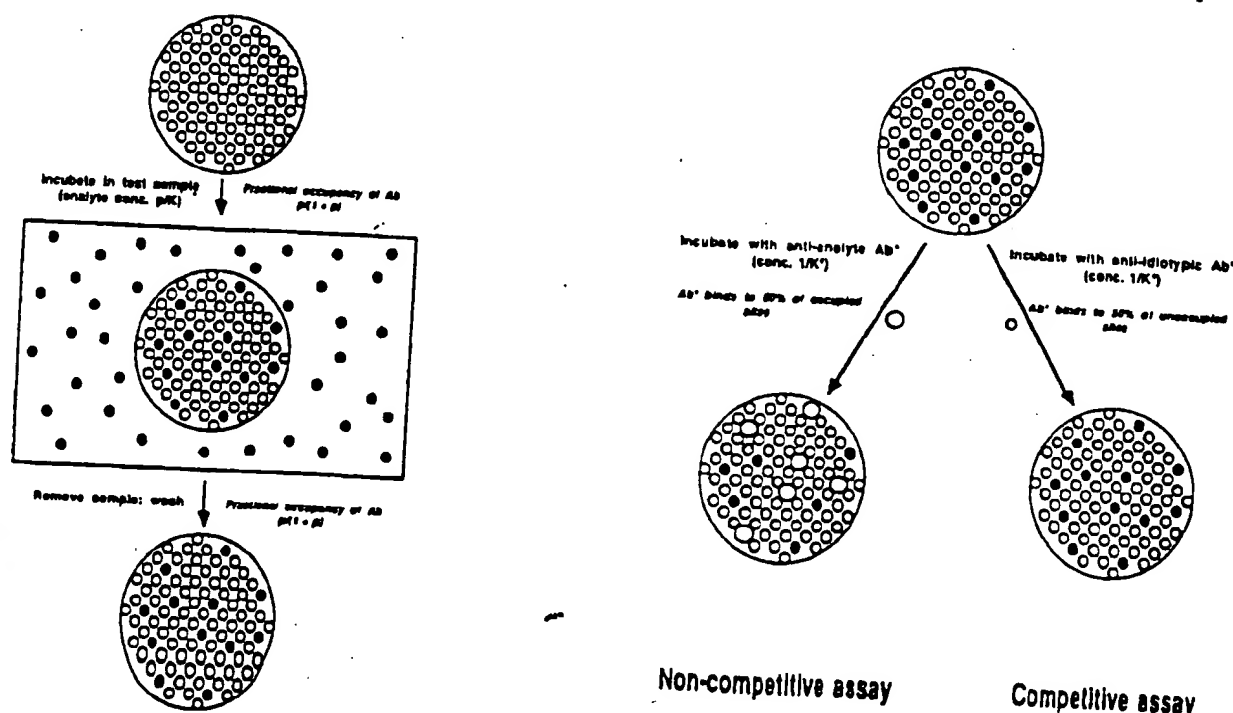


Fig. 8. Microspot Immunoassay: (left) first incubation, with the fractional occupancy of antibody binding sites reflecting the analyte concentration to which the microspot has been exposed; (right) second incubation, in which the microspot is exposed to a second "developing" antibody reactive with either occupied sites (noncompetitive assay), or unoccupied sites (competitive assay). In the second incubation, a concentration of developing antibody has been selected such that only 50% of the occupied or unoccupied sites is identified

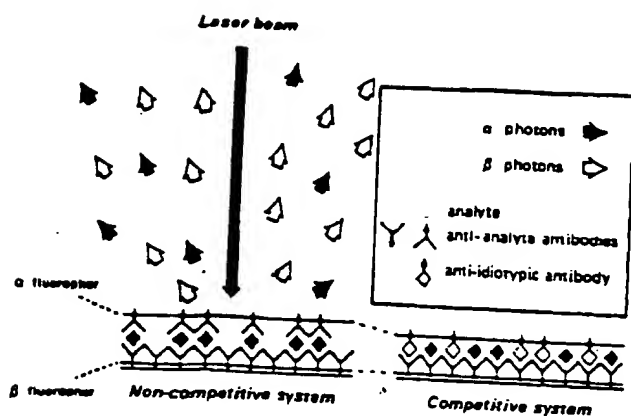


Fig. 9. Basic principle of dual-label, ambient analyte immunoassay relying on fluorescent-labeled antibodies

The ratio of  $\alpha$  and  $\beta$  fluorescent photons emitted reflects the value of  $F$  (see Fig. 7) and depends solely on the analyte concentration to which the probe has been exposed. The ratio is unaffected by the amount or distribution of antibody coated (as a monomolecular layer) onto the probe surface

advantages stem from adopting a dual fluorescence measurement. For example, neither the amount nor the distribution of the sensor antibody within the detector's field of view is important, because the ratio of the emitted fluorescent signals is unaffected. Likewise, fluctuations in the intensity of the incident (exciting) light beam are apt to be of little significance. These advantages are additional to the basic benefit stemming from this approach, i.e., that the necessity of ensuring constancy of the amount of sensor antibody used in the assay system is removed.

#### Microspot Immunoassay Sensitivity

Because the microspot immunoassay methodology challenges concepts that have dominated immunoassay design theory in the past two to three decades, consideration of the potential sensitivity attainable by this approach is obviously of primary importance. The proposition that microspot assays may be at least as sensitive as conventional systems that rely on far larger amounts of antibody may readily be demonstrated by consideration of a model system. Let us postulate that sensor antibody molecules are attached to the surface of a solid support such that their binding sites remain exposed to the analyte, and that their affinity for the analyte is thereby unchanged. (The antibody concentration in the system—the number of binding sites on the support divided by the incubation volume—is unaffected by such attachment, and antibody occupancy by analyte at equilibrium will be identical to that occurring if the antibody is distributed uniformly throughout the incubation mixture.) Let us also suppose that the antibody molecules exist as a uniform monolayer of maximal surface density on the support and (to simplify discussion) are unlabeled. Then a change in the concentration of sensor antibody implies a corresponding change in the surface area over which the antibody is distributed. If, for example, the antibody affinity constant is  $10^{11}$  L/mol, the total incubation volume is 1 mL, and the antibody surface density is 6000 binding sites/ $\mu\text{m}^2$ , then

a surface area of  $10^5 \mu\text{m}^2$  (i.e.,  $0.1 \text{ mm}^2$ ) accommodates antibody binding sites corresponding to a concentration of  $0.1/K$ ; an area of  $0.01 \text{ mm}^2$  corresponds to a concentration of  $0.01/K$ , etc. Let us further postulate that, after exposure of the sensor antibodies to a medium containing analyte at a concentration of  $0.01/K$  (i.e.,  $6 \times 10^7$  molecules/mL), we measure "noncompetitively" the resulting antibody occupancy (e.g., by exposure to a second, labeled, "developing" antibody directed against the analyte, forming a typical antibody sandwich). Finally, let us suppose that all occupied sites react with the developing antibody, with the latter also binding "non-specifically" to the solid support itself at a surface density of 1 molecule/ $\mu\text{m}^2$ .

We may now consider the effects of a progressive reduction of the antibody-coated surface area from (e.g.)  $1 \text{ mm}^2$  (effective antibody concentration  $1/K$ ) through  $0.1 \text{ mm}^2$  ( $0.1/K$ ) to  $0.01 \text{ mm}^2$  ( $0.01/K$ ) and below. From equation 4, the value of  $F$  for the  $1 \text{ mm}^2$  area is  $4.98 \times 10^{-3}$ . Thus at equilibrium the number of analyte and labeled antibody molecules specifically bound to the area is  $2.99 \times 10^7$  (i.e., about 50% of the total analyte molecules present), whereas the number of labeled antibody molecules nonspecifically bound is  $10^6$ . Thus, assuming the field of view of the detecting instrument is restricted to the area on which the sensor antibody is deposited (see Figure 10a), and (provisionally) assuming the background (or "noise") of the instrument itself to be zero (i.e., the only source of background is the non-

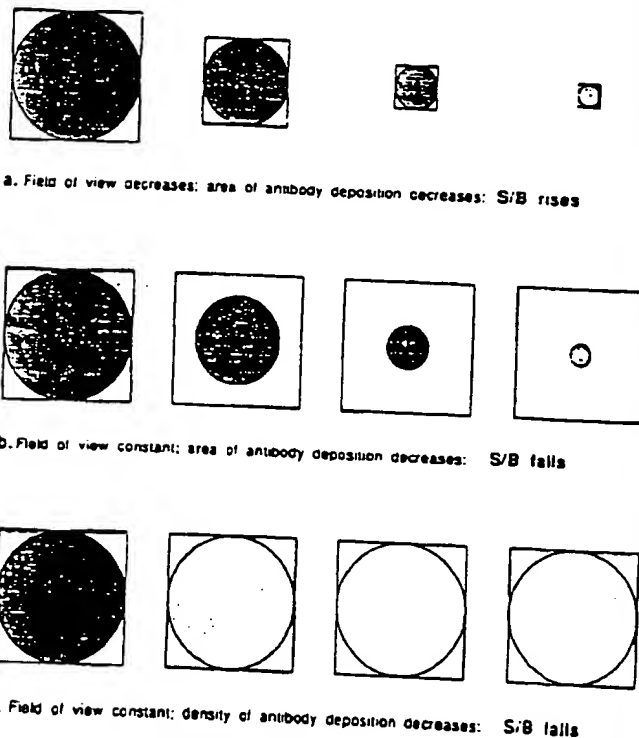


Fig. 10. "Capture" antibody (CAB) is assumed coated on circular (shaded) areas; the field of view of the signal-measuring instrument is represented by square (unshaded) areas

(a) Reduction of both the area of deposition of CAB and the field of view results in an increase in the signal/noise (S/B) ratio. If the CAB is reduced either by reducing the antibody coated area (b) or the density of antibody coating (c) while the field of view remains unchanged, S/B falls



specifically-bound labeled antibody within the instrument's field of view), the signal/noise ratio observed for the 1 mm<sup>2</sup> area is ~30. Similarly, the value of  $F$  for a 0.1 mm<sup>2</sup> area is  $9.02 \times 10^{-3}$ , the number of labeled antibody molecules specifically bound to the area is  $5.41 \times 10^6$ , the number nonspecifically bound is  $10^6$ , and the signal/noise ratio is ~54. Likewise, the signal/noise ratio for a 0.01 mm<sup>2</sup> area can be shown to be ~59. In short, the signal/noise ratio increases as the antibody-coated surface area is decreased, approaching a maximal (plateau) value of 60 as the area coated with sensor antibody falls below 0.01 mm<sup>2</sup> and tends toward zero.

If, however, a reduction in the antibody-coated area were not accompanied by a corresponding reduction in the detecting instrument's field of view, the resulting reduction in "signal" would not lead to a corresponding decrease in the background generated by nonspecifically-bound developing antibody (Figure 10b). Therefore, although reduction in the coated area would increase the fractional occupancy of the sensor antibody, the signal/noise ratio might either remain constant or fall. In these circumstances it might be advantageous to increase the coated area. Similarly, if the surface density of sensor antibody were decreased (the coated area being held constant), similar conclusions would be reached (Figure 10c).

Likewise, if the background signal generated within the detecting instrument itself (e.g., from the photocathode of a photomultiplier tube used to detect photons emitted from the antibody-coated area) were not zero, and remained constant regardless of the instrument's field of view, then a maximum signal/noise ratio would also be attained at some optimal value of the antibody-coated area, below which the ratio would fall. Because, however, one can generally reduce the size of the detector (and hence the detector-generated background) at the same rate as the size of the signal-emitting area, there is no reason—in principle—for the signal/noise ratio to diminish as the antibody-coated area is progressively reduced toward zero. Thus if we accept the signal/noise ratio as indicative of the precision of the measurement of antibody occupancy (and hence of assay sensitivity), these considerations suggest that it is advantageous to reduce the antibody-coated surface area (and, concomitantly, the sensor-antibody concentration) toward zero, although little advantage is likely to accrue from reducing the area below 0.01 mm<sup>2</sup> (and thus the antibody concentration below 0.01/K).

Were the microspot area indeed reduced to zero, both signal and noise would likewise also fall to zero (the ratio between them nevertheless remaining essentially constant), implying that no signal of any kind would, in the limit, be recorded. In practice, other statistical factors come into play when the number of individual events (e.g., photons) observed by a detecting instrument is very low, thus prohibiting a reduction of the sensor antibody concentration to zero. The point at which the reduction in the antibody-coated area causes the detectable signal to be lost sufficiently to affect the

precision of the measurement of antibody occupancy depends clearly on the specific activity of the labeled antibody used to measure the occupied binding sites: the higher the specific activity, the smaller the permissible area. Thus, given labels of very high specific activity, one can envision circumstances in which, even in a "noncompetitive" system, the optimal concentration of sensor antibody may be exceedingly low. A more general conclusion is that a variety of factors, including the characteristics of the instruments used for measuring the labeled antibody (or labeled analyte), influence immunoassay design, implying, among other things, the virtual impossibility of formulating general rules regarding this. For example, reagent concentrations that are optimal for isotopically labeled reagents used with a conventional radioisotope counter (possessing a fixed background dependent on its basic construction) are likely to be entirely different when very high-specific-activity labels are used and one has the freedom to tailor the measuring instrument to samples of any size. In short, certain conclusions based on experience of RIA and IRMA techniques may prove misleading when applied to nonisotopic methodologies, and should be viewed with caution.

A more detailed theoretical consideration of (noncompetitive) microspot immunoassay sensitivity (21) suggests that

$$C_{\min} = D^*_{\min} \times [(6 \times 10^{20})(1 + [Ab^*])/DK[Ab^*]] \quad (5)$$

where  $D$  = surface density (binding sites/ $\mu\text{m}^2$ ) of sensor antibody,  $K$  = sensor antibody affinity (L/mol),  $[Ab^*]$  = concentration of labeled antibody in developing solution (expressed in units of  $1/K^*$ , where  $K^*$  = labeled antibody affinity),  $D^*_{\min}$  = minimum detectable surface density of labeled antibody (molecules/ $\mu\text{m}^2$ ), and  $C_{\min}$  = assay detection limit (molecules/mL). For example, if  $[Ab^*] = 1$ ,  $D = 10^6$  molecules/ $\mu\text{m}^2$ ,  $K = 10^{11}$  L/mol, and  $D^*_{\min} = 20$  molecules/ $\mu\text{m}^2$ , then  $C_{\min} = 2.4 \times 10^6$  molecules/mL =  $4 \times 10^{-16}$  mol/L and the fractional occupancy of the binding sites of the sensor antibody by the minimum detectable concentration of analyte is 0.04%. Figure 11 shows the theoretical assay sensitivities attainable with use of sensor antibodies of various affinities, plotted as a function of  $D^*_{\min}$ .

A similar theoretical analysis of competitive microspot immunoassay indicates that potential sensitivities are essentially identical to those attainable with conventional competitive methodologies. In summary, the above considerations indicate that the attainment of high microspot assay sensitivity requires close packing of molecules of sensor antibodies within the microspot area, combined with the use of an instrument capable of accurately measuring very low surface densities of developing antibodies. They also suggest that (a) microspot assay sensitivities considerably higher than those obtainable by conventional isotopically based immunoassays are achievable, and (b) if labels of very high specific activity are available, the sensitivities yielded

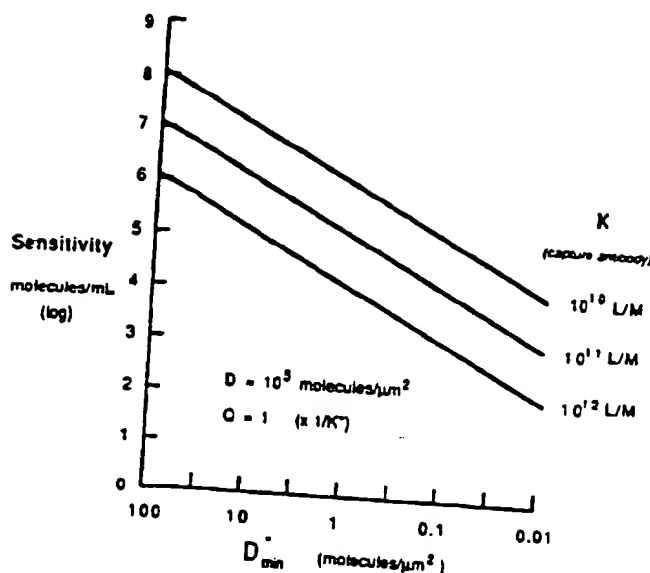


Fig. 11. Theoretically predicted sensitivity of noncompetitive microspot immunoassay plotted as a function of the minimum developing antibody density detectable within the microspot area

Postulated values of capture antibody surface density are  $10^5$  molecules/ $\mu\text{m}^2$ , and of developing antibody concentration are  $1/K$ . Currently available instruments permit detection of between 10 and 1 molecules of fluorescein-labeled antibody per micrometer<sup>2</sup>

by microspot assays are unlikely to be inferior and (depending on the characteristics of the measuring instruments used) could be superior to the sensitivities achievable in macroscopic assays of conventional design.

Finally, we briefly address a further question occasionally raised in this context, i.e., the kinetic characteristics of microspot assays. Two points should be made regarding this issue. First, the smaller the microspot of sensing antibody, the lower the diffusion constraints on the velocity of the antibody/analyte binding reaction, so that at the limit (i.e., when the amount of antibody situated within the microspot area approaches zero) the kinetics of the reaction approximate those observed in a homogeneous liquid-phase system. Second, although the effective concentration of sensor antibody in the incubation medium is exceedingly low, the fractional rate at which sensor antibody binding sites within the microspot become occupied is invariably greater in this circumstance than when a relatively high concentration of antibody is used, as in conventional assays, particularly those of noncompetitive design. In other words, bearing in mind the relationship between fractional occupancy of sensor antibody and the signal/noise ratio discussed above, it is readily demonstrable that the rate at which the ratio rises is greatest when the microspot area (and the antibody contained within it) is least. Thus, given instrumentation whose field of view is restricted to the microspot area, the highest signal/noise ratio will be observed (after any selected incubation period) when the concentration of sensor antibody in the system is  $<0.01/K$ . In short, contrary perhaps to superficial impression, and to the generally accepted belief that short immunoassay incubation times require the use of very large amounts of antibody, the antibody microspot ap-

proach provides the basis of assays potentially more rapid than any currently available.

#### Microspot Immunoassay: Some Practical Considerations

Although various high-specific-activity antibody labels are potentially usable in this context, our preliminary studies have relied on the use of conventional fluorophors. The simultaneous measurement of dual fluorescences from small areas is, of course, well established, and the availability of improved instrumentation (e.g., the laser scanning confocal microscope), albeit not specifically designed for the present purpose, has been useful in demonstrating the feasibility of the microspot approach.

In laser scanning confocal fluorescence microscopes, a small area of the specimen is illuminated by a focused laser beam, the fluorescence photons emitted from this area being focused in turn onto a detector, typically a low-dark-current photomultiplier (22, 23). At the "confocal" point, the projection of the illumination pinhole and the back-projection of the detector pinhole coincide (Figure 12). Fluorescence photons emitted at other points thus possess a low probability of reaching the detector. Such systems contrast with conventional epifluorescence microscopes, in which the specimen is exposed to an essentially uniform flux of illumination, and yield much sharper images of fluorescent emitters situated in a defined plane of a tissue sample. Electrons spontaneously emitted by the photomultiplier photocathode contribute to the background signal of the instrument, and must—for highest microspot assay sensitivity—be minimized. Fortunately, the design of such instruments permits the photocathode to be very small in area, and this source of background can be expected

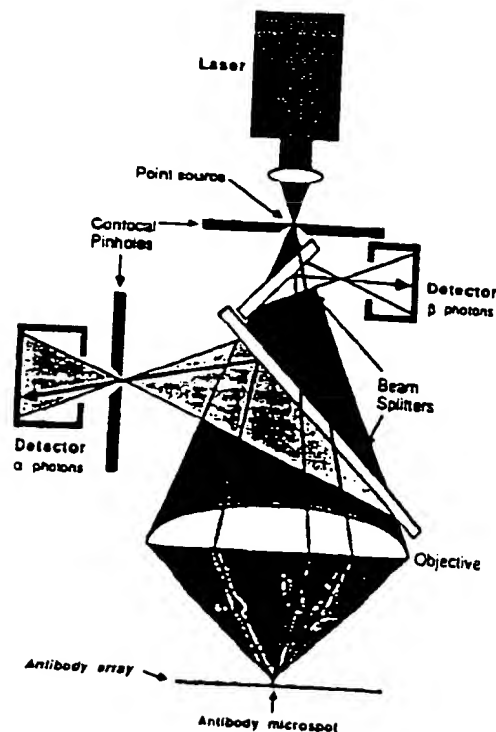


Fig. 12. Schematic diagram of a dual-channel confocal microscope

to diminish with future improvement in photomultiplier design. Other sources of background include fluorescence emitted by components in the optical system, which may not, in current instruments, have been constructed with background reduction as a prime consideration. Nevertheless, they detect with high sensitivity fluorescent signals. For example, one commercially available microscope is claimed to detect fluorescein at a density of 10 molecules/ $\mu\text{m}^2$ . Most commercially available fluorescein isothiocyanate (FITC)-labeled IgG exhibits a fluorophor/protein ratio of  $\sim 4$ ; this implies detection limit ( $D^*_{\text{min}}$ ) for antibody surface density of two or three FITC-labeled IgG molecules per micrometer<sup>2</sup>. This, in turn, implies a theoretical sensitivity for a two-site immunoassay of  $\sim 2\text{--}3 \times 10^5$  analyte molecules per milliliter, assuming identical parameter values as above, or  $2\text{--}3 \times 10^4$  molecules/mL if the sensing antibody has an affinity of  $10^{12}$  L/mol. Clearly, sensitivity may be increased by loading more fluorophor either directly or indirectly onto the antibody.

Our preliminary studies have relied on a less sensitive microscope, albeit one possessing facilities for dual-fluorescence measurement. Its argon laser emits two excitation lines at 488 and 514 nm. It is thus particularly efficient in exciting blue/green-emitting fluorophores such as FITC (excitation maximum 492 nm), but is less efficient in exciting fluorophores such as Texas Red (excitation maximum 596 nm). However, the ratiometric assay principle permits considerable variation in detection efficiencies of the two labels because the specific activities of the labeled antibody species forming the antibody couplets can be chosen to yield signal ratios approximating unity. Inefficiency of the argon laser in exciting Texas Red is thus not a major handicap in this context. Though this instrument relies on a conventional microscope and not on an optical system designed for this purpose (and thus implicitly less sensitive), it permits quantification of fluorescence signals generated from microspots of any selected area. Initial studies have revealed that, under conditions that are not optimal, the instrument is capable of detecting  $\sim 25$  FITC-labeled and (or) 150 Texas Red-labeled IgG molecules per micrometer<sup>2</sup>, while scanning an area of  $\sim 50 \mu\text{m}^2$ .

The development of microspot immunoassays has also necessitated closer scrutiny of the mechanisms involved in the coupling of antibodies to solid supports. In the present context, these should display a capacity to adsorb (in the form of a monolayer)—or to covalently link—a high surface density of antibody combined with low intrinsic-signal-generating properties (e.g., low intrinsic fluorescence), thus minimizing background. We have examined a number of candidate materials, such as polypropylene, Teflon®, cellulose and nitrocellulose membranes, microtiter plates (clear polystyrene plates; black, white, and clear polystyrene plates), glass slides and quartz optical fibers coated with 3-(amino propyl) triethoxy silane, etc., and several alternative protocols for achieving high monolayer coating densities. These

studies have exposed phenomena neither evident nor of importance when antibody binding to solid supports is examined at a macroscopic level. Provisionally, we have used white Dynatech Microfluor microtiter plates—formulated for the detection of low fluorescence signals, and yielding high signal/noise ratios and high coating densities of functional antibodies ( $\sim 5 \times 10^4$  IgG molecules/ $\mu\text{m}^2$ )—for assay development, although such plates are not ideal. Indeed, deficiencies in the antibody-deposition methods used constitute the principal source of imprecision in assay results and the limitation in sensitivity that this implies. Clearly, this represents an area for further study and refinement of current coating techniques.

Notwithstanding the limitations of present instrumentation (which, among other things, does not permit the use of time-resolving techniques to distinguish two individual fluorescence signals either from each other or from background fluorescence) and the crudeness of present methods for coupling antibodies onto small areas, we have verified the theoretical concepts outlined above by comparing the performance of several assays when constructed in microspot format and when conventionally designed. Although unoptimized, ratiometric microspot assays have yielded sensitivity values closely approaching those of conventional optimized IRMA. As an example, the results of a ratiometric assay system for thyrotropin, with use of Texas Red- and FITC-labeled antibodies, are shown in Figure 13. Bearing in mind the well-known limitations of these and other "conventional" fluorophors when used as immunoassay reagent labels, such results are encouraging, although further work is clearly required to achieve the considerably greater sensitivity theoretically predicted with use of improved fluorophors, better antibody-microspotting techniques, and purpose-built (time-resolving) instrumentation.

The finding that highly sensitive immunoassays can be performed with far smaller amounts of antibody than

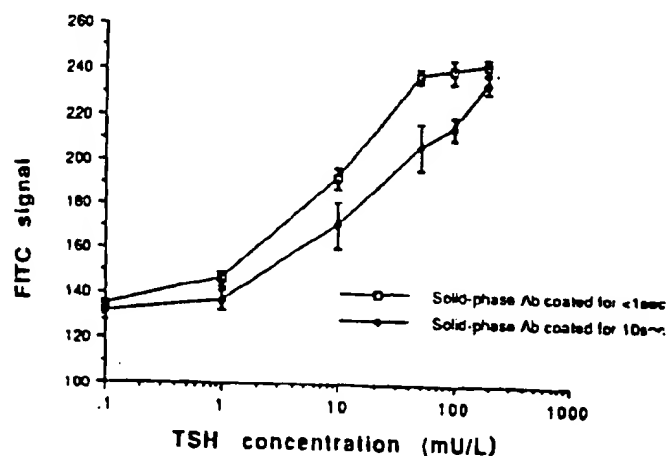


Fig. 13. Response curve in a dual-labeled microspot ratiometric assay of thyrotropin (TSH) with Texas Red-labeled solid-phase capture antibody and a developing antibody labeled with biotin/FITC-avidin

The FITC/Texas Red ratio for each microspot was measured with a scanning confocal microscope, and plotted as a function of TSH concentration in milli-int. units/L

are currently used conventionally permits in turn the construction of antibody microspot arrays enabling, in principle, the simultaneous measurement of thousands of different substances in 1-mL samples. In collaboration with investigators at the Centre for Applied Microbiological Research, Porton Down, U.K., we are presently developing various techniques for the creation of such arrays. Indeed, similar technologies have recently been used for the parallel synthesis of several different polypeptides, these enabling 10 000-microspot arrays to be constructed on silica chips approximating 1 cm<sup>2</sup> (24). Although arrays of this capacity are unlikely to ever be required for conventional diagnostic purposes, we can anticipate that the ability to simultaneously measure many substances in the same sample will have revolutionary consequences in medicine and other similar areas. In addition, such techniques may ultimately permit the individual analysis of the multiple isoforms of certain "heterogeneous" analytes (e.g., the glycoprotein hormones), such molecular heterogeneity currently presenting a major obstacle to the standardization and interpretation of many immunological measurements (25). Moreover, although these concepts have been illustrated in an immunoassay context, they are clearly applicable to all "binding assays," including those relying on the use of DNA probes, hormone receptors, etc. For example, labeled lectins that are specific in their reactions with the sugar residues in the oligosaccharide chains of glycoprotein molecules may be used, together with specific antibodies, to impart additional "structural specificity" to sandwich assays (26, 27), possibly overcoming the limitations of antibodies per se in regard to differentiation of the glycosylation variants of the glycoprotein hormones.

### Summary and Conclusion

Because of past confusion regarding the concepts of precision, sensitivity, accuracy, etc., several erroneous concepts have become incorporated within currently accepted rules of immunoassay design. In particular, much higher antibody concentrations are customarily used than are necessary to achieve very high assay sensitivity, provided that certain measurement strategies are adhered to. In this presentation, we have attempted to show that, in principle, the highest assay sensitivities are obtained by confining a small number of sensor antibody molecules onto a very small area in the form of a microspot and measuring their occupancy by an analyte, by using very high-specific-activity "developing" antibody probes, thereby maximizing the signal/noise ratio in the determination of sensor antibody occupancy. This observation, which contradicts currently accepted immunoassay design theory, in turn makes possible the measurement of an unlimited number of different analytes on a chip of very small surface area through the use of, e.g., laser scanning techniques closely analogous to those used in compact disk techniques of sound recording. Extensive experimental studies in this area, albeit conducted with relatively crude techniques and instrumentation not specifically de-

signed for these purposes, and therefore not reported in detail here, have demonstrated the feasibility of the miniaturized antibody microspot approach and the validity of the general concepts on which it is based. We are therefore confident that this represents the basis of a next-generation technology that is likely to have a revolutionary impact on all fields involving the use of binding assays.

### References

1. Yalow RS, Berson SA. General principles of radioimmunoassay. In: Hayes RL, Goswitz FA, Murphy BEP, eds. *Radioisotopes in medicine: in vitro studies*. Oak Ridge, TN: US Atomic Energy Commission, 1968:7-39.
2. Ekins RP, Newman B, O'Riordan JH. *Ibid.*: 59-100.
3. Berson SA, Yalow RS. Measurement of hormones—radioimmunoassay. In: Berson SA, Yalow RS, eds. *Methods in investigative and diagnostic endocrinology*, Vol. 2A. Amsterdam: North Holland/Elsevier, 1973:84-135.
4. Ekins R, Newman B. Theoretical aspects of saturation analysis. In: Diczfalussy E, Diczfalussy A, eds. *Steroid assay by protein binding*. Karolinska symposium on research methods in reproductive endocrinology. Stockholm: WHO/Karolinska Sjukhuset, 1970:11-30.
5. Ekins RP. Limitations of specific activity. In: Margoulies M, ed. *Protein and polypeptide hormones, Part 3 (Discussions)*. Amsterdam: Excerpta Medica, 1968:612-6, et seq.; Ekins RP. Concentrations of tracer and antiserum, time and temperature of incubation, volume of incubation. *Ibid.*: 672-82.
6. Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960;39:1157.
7. Ekins RP. The estimation of thyroxine in human plasma by an electrophoretic technique. *Clin Chim Acta* 1960;5:453-9.
8. Barakat RM, Ekins RP. Assay of vitamin B<sub>12</sub> in blood—a simple method. *Lancet* 1961;ii:25-6.
9. Wide L, Bennich H, Johansson SGO. Diagnosis of allergy by an in-vitro test for allergen antibodies. *Lancet* 1967;ii:1105-7.
10. Miles LEH, Hales CN. Labeled antibodies and immunological assay systems. *Nature (London)* 1968;219:186-9.
11. Keston AS, Udenfriend S, Cannan RK. Micro-analysis of mixtures (amino acids) in the form of isotopic derivatives. *J Am Chem Soc* 1946;68:1390.
12. Avivi P, Simpson SA, Tait JF, Whitehead JK. The use of <sup>3</sup>H and <sup>14</sup>C-labeled acetic anhydride as analytical reagents in micro-biochemistry. In: Johnston JE, Faires RA, Millett RJ, eds. *Radioisotope conference*. London: Butterworths, 1954:313-23.
13. Miles LEH, Hales CN. An immunoradiometric assay of insulin. *Op. cit.* (ref. 5), Part 1:61-70.
14. Rodbard D, Weiss GH. Mathematical theory of immunometric (labeled antibody) assay. *Anal Biochem* 1973;52:10-44.
15. Jackson TM, Marshall NJ, Ekins RP. Optimisation of immunoradiometric assays. In: Hunter WM, Corrie JET, eds. *Immunoassays for clinical chemistry*. Edinburgh: Churchill Livingstone, 1983:557-75.
16. Ekins RP. Measurement of analyte concentration. British patent no. 8 224 600, 1983.
17. Wide L. Solid-phase antigen-antibody systems. In: Hunter WM, Kirkham KE, eds. *Radioimmunoassay methods*. Edinburgh: Churchill Livingstone, 1971:405-12.
18. Köhler G, Milstein C. Continuous culture of fused cells secreting specific antibody of predefined specificity. *Nature (London)* 1975;256:495-7.
19. Marshall NJ, Dakubu S, Jackson T, Ekins RP. Pulsed light, time resolved fluoroimmunoassay. In: Albertini A, Ekins RP, eds. *Monoclonal antibodies and developments in immunoassay*. Amsterdam: Elsevier/North Holland, 1981:101-8.
20. Soini E, Lövgren T. Time-resolved fluorescence of lanthanide probes and applications in biotechnology [Review]. *Crit Rev Anal Chem* 1987;18:105-54.
21. Ekins RP, Chu F, Biggart E. The development of microspot, multi-analyte radioimmunoassay using dual fluorescent-labeled antibodies. *Anal Chim Acta* 1990;227:73-96.
22. White JG, Amos WB, Fordham M. An evaluation of confocal versus conventional imaging of biological structures by fluores-

cence light microscopy. *J Cell Biol* 1987;105:41-8.

23. Ploem JS. New instrumentation for sensitive image analysis of fluorescence in cells and tissues. In: Tayer DL, Waggoner AS, Lanni F, Murphy R, Birge R, eds. *Applications of fluorescence in the biological sciences*. New York: Alan R Liss, 1986:289-300.

24. Fodor SPA, Read JL, Pirrung MC, et al. Light-directed, spatially addressable parallel chemical synthesis. *Science* 1991;251:767-73.

25. Ekins RP. Immunoassay standardization. In: Kallner A, Magid E, Albert W, eds. *Improvement of comparability and compatibility of laboratory assay results in life sciences*. Immunoassay standardization. *Scand J Clin Lab Invest* 1991;51(Suppl 205):33-46.

26. Kottgen E, Hell B, Muller C, Tauber R. Demonstration of glycosylation variants of human fibrinogen, using the new technique of glycoprotein lectin immunosorbent assay (GLIA). *Biol Chem Hoppe Seyler* 1988;369:1157-66.

27. Kinoshita N, Suzuki S, Matsuda Y, Taniguchi N.  $\alpha$ -Fetoprotein antibody-lectin enzyme immunoassay to characterise sugar chains for the study of liver diseases. *Clin Chim Acta* 1989;179:143-52.

28. Shalev V, Greenberg GH, McAlpine PJ. Detection of antigens by a high sensitivity enzyme-linked immunosorbent assay (HS-ELISA) using a fluorogenic substrate. *J Immunol Methods* 1980;88:125.

29. Harris CC, Yolken RH, Kroken H, Hsu IC. Ultrasensitive enzymatic radioimmunoassay: application to detection of cholera toxin and rotavirus. *Proc Natl Acad Sci USA* 1979;76:5336.

## Corrections

Vol 37, pp. 1447-8: In our desire for rapid publication, important errors were introduced into the following Technical Brief. The corrected version is here reproduced in its entirety, with our apologies to the authors.

**Rapid Detection of 1717-1G→A Mutation in CFTR Gene by PCR-Mediated Site-Directed Mutagenesis**, Laura Cremonesi,<sup>1</sup> Manuela Seia,<sup>2</sup> Carmelina Magnani,<sup>1</sup> and Maurizio Ferrari<sup>1</sup> (<sup>1</sup> Istituto Scientifico H.S. Raffaele, Lab. Centrale, Milano; <sup>2</sup> Istituti Clin. di Perfezionamento, Lab. di Ricerche Clin., Milano, Italy)

Until now, among the non- $\Delta F508$  mutations identified in the cystic fibrosis transmembrane conductance regulator (CFTR) gene by the Cystic Fibrosis (CF) Genetic Analysis Consortium, the ones most frequently seen in our population sample are the 1717-1G→A mutation (13/144 or 9% of the CF chromosomes) and the G542X mutation (16/190 or 8.4% of the CF chromosomes), both revealed by dot-blot hybridization of the polymerase chain reaction (PCR) product with allele-specific oligonucleotides (ASO) probes (1).

In an attempt to simplify the analysis of the most frequent mutations in the CFTR gene, we converted radio-labeled ASO detection into restriction endonuclease analysis of the amplified product.

A PCR-mediated site-directed mutagenesis (2, 3) to detect the G542X mutation by generating a novel *Bst*NI site in the wild-type sequence had already been suggested (4).

To detect the 1717-1G→A mutation, we designed the reverse primer (5'-CTCTGCAAACCTGGAGAGGTC-3') to contain a single-base mismatch (T→G), which could create a novel *Ava*II restriction site [G ↓ G(A/T)CC] in the amplified wild-type (WT) allele but not in the CF mutant (M) allele:

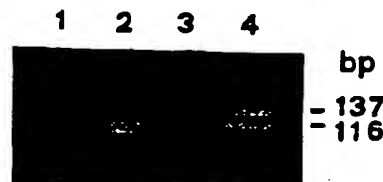
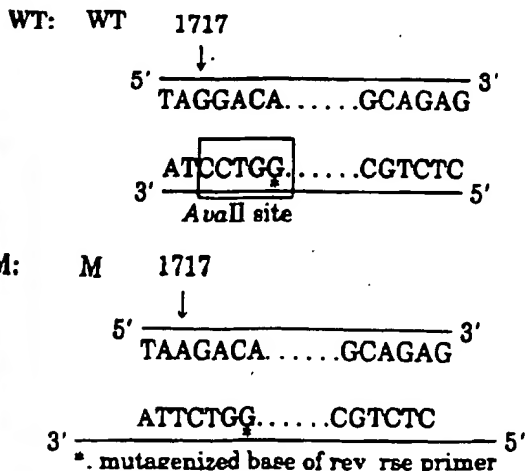


Fig. 1. Detection of the 1717-1G→A mutation by PCR

Reactions were carried out with 1  $\mu$ g of genomic DNA in a total volume of 100  $\mu$ L containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.6 mmol/L  $MgCl_2$ , 0.1 g/L gelatin, 200  $\mu$ mol/L each of the four deoxyribonucleotide triphosphates, 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 100 pmol of each of the primers. PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, for a total of 30 cycles. PCR products were digested for 2 h at 37 °C with 5 U of *Ava*II and electrophoresed on 3% agarose-1% NuSieve gel for 1 h at 50 V. Bands were made visible by staining the gel with ethidium bromide. Lane 1: *Hae*III-digested pBR322 size marker. Lane 2: normal homozygote. Lane 3: CF patient homozygous for the 1717-1G→A mutation. Lane 4: heterozygote carrier for the 1717-1G→A mutation

For the forward primer, we used the one made available by the CF Genetic Analysis Consortium to amplify exon 11 of the CFTR gene: 5'-CAACTGTGGTTAAAGCAAT-AGTGT-3'.

Digestion by *Ava*II enzyme of the PCR product generates two fragments of 116- and 21-bp in the wild-type alleles and leaves undigested a 137-bp fragment in the mutant alleles (Figure 1).

By combined analysis for the  $\Delta F508$  mutation (5) (252/470 or 53.6% of the CF chromosomes), 1717-1G→A, and G542X, about 71% of mutations might be detected by nonisotopic analysis of the PCR product, thus allowing a faster and easier one-day procedure for carrier screening and prenatal testing.

## References

1. Kerem B, Zielenski J, Markiewicz D, et al. Identification of mutations in regions corresponding to the two putative nucleotide (ATP)-binding folds of the cystic fibrosis gene. *Proc Natl Acad Sci USA* 1990;87:8447-51.
2. Haliassos A, Chomel JC, Baudis M, Kruh J, Kaplan JC, Kitzis A. Modification of enzymatically amplified DNA for the detection of point mutations. *Nucleic Acids Res* 1989;17:3606.
3. Friedman WE, Highsmith E Jr, Prior TW, Perry TR, Silverman LM. Cystic fibrosis deletion mutation detected by PCR-mediated site-directed mutagenesis [Tech Brief]. *Clin Chem* 1990;36:695-6.
4. Ng ISL, Pace R, Richard MV, et al. Methods for analysis of multiple cystic fibrosis mutations. *Hum Genet* (in press).
5. Ferrari M, Cremonesi L. More on detection of cystic fibrosis by polymerase chain reaction [Response to Letter]. *Clin Chem* 1990;36:1702-3.



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11  
91

## In This Issue . . .

Komberg on Life as Chemistry

See Page 1895

Cyclosporine Monitoring

See Pages 1891, 1905

Clinical Uses of DNA Amplification

See Pages 1893, 1945, 1983

CLIA and Cholesterol Testing

See Page 1938

American Thyroid Association  
Report

See Page 2002

